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VITAMIN A

I. The Visual Process

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VITAMIN A
I. The Visual Process

By W. A. KREHL, Ph.D. *(Guest Editor)**

Introduction

In general, the periods which characterize the history of a vitamin are concerned first with the recognition of a disease entity caused by the lack or deficiency of the factor; second, by the isolation, chemical identification and synthesis of the substance, and, finally, by the elucidation of its specific role in the reactions involved in the normal physiology of the organism.

The combined zeal and enthusiasm with which the nutritionist, chemist, biochemist and clinician have approached the field of vitamin study has resulted in a long list of both water and fat soluble vitamins and an extensive application of these substances in various phases of nutrition and medicine.

On the other hand the clarification of the specific biochemical roles of these vital catalysts, the vitamins, has failed to keep pace with the more general developments.

When the subject of vitamin A and vision was first presented in this journal (1), the review foreshadowed the results that were to follow. The expanded knowledge concerning the role of vitamin A in the visual process has resulted primarily from the work of George Wald and his colleagues and has placed the biochemical role of this vitamin on so much firmer ground that it deserves to be reviewed again.

Vitamin A Deficiency

The classical studies of McCollum and Davis and Osborne and Mendel which led to the discovery of vitamin A are well known, as are the principal signs and symptoms of a deficiency of this vitamin (1, 2, 3).

Vitamin A deficiency in man may be due to a direct lack of this factor in the diet, or to an inability to absorb, retain, or release the vitamin from its physiological storage sites. Although frank vitamin A deficiency is considered uncommon in this country, a considerable proportion of the population, according to dietary surveys, does not receive the recommended amounts of vitamin A.

Night Blindness

Night blindness, or "hemeralopia," is the inability to see in dim light, although vision in bright light is unimpaired. The first symptom of night

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blindness may be noticed after exposure to brilliant light followed by an inability to adapt the visual process to a dark environment. Evidence for the existence of this disease dates back to antiquity (1). The potential dangers of night blindness are best appreciated when one considers how important it is to be able to "see again" after having been "blinded" by the bright lights of approaching automobiles; or the need of the airplane pilot to have good vision in dim light.

Since the symptom of night blindness is one of the first seen in vitamin A deficiency, the measurement of dark adaptation is very useful in nutritional research on human subjects. Such measurements are much more easily made and perhaps have more practical meaning than the determination of the vitamin A level of the blood.

It must be remembered however, that other conditions beside a deprivation of vitamin A may impair dark adaptation. These are best ruled out if distinct and rapid improvement results after giving vitamin A.

The Eye as a Camera

Wald (4) has stated that "of all the instruments made by man none resembles a part of his body more than a camera does the eye." To illustrate, both the iris of the camera and the pupil of the eye are opened more in dim light for the purpose of passing more light energy through the lens so that the image can better register on the retina of the eye or the sensitive emulsion of the film. As the light becomes even more dim, the photographer must turn to a film emulsion of greater sensitivity which is coarser in grain. In general, the combined procedures of using a wider lens opening and a more sensitive film in dim light result in pictures which lack sharpness of image and depth of field.

Just as the photographic film has light sensitive crystals of silver bromide, the retina of the eye is composed of an intricate network of light-sensitive receptor cells called the rods and the cones.

The general distribution of the rods and cones over the surface of the retina is such as best to accommodate the intensity of light admitted through the lens by the pupil of the eye. The center of the retina is occupied by cones which are the cells responsible for vision in bright light and also for color vision. The rest of the retina contains mostly rods which become increasingly predominant near the periphery of the retina. The rods then provide the means by which vision is possible in dim light. The stimulation of the rods by the energy of dim light results in "neutral gray sensations" — these are not the cells of color vision. Just as the cones are the cells analogous to the slow fine-grained film which permits a high quality, sharply defined image,

the rods correspond to the slow, highly sensitive coarse-grained film which resolves the image less accurately. Another factor involved in the reduced image — resolving capacity of the rods depends on the fact that whereas each individual cone is connected with the brain through a simple nerve fiber of the optic nerve, large groups or clumps of rods are connected by single nerve fibers.

Dim light results in stimulation of the rods, and the cones are not involved. As light intensities increase, both rods and cones function until in bright light the cones dominate the visual process. At low light intensities, sacrifices are made in the capacity to resolve the image or see what color it is. In bright light the pupil narrows, vision is shifted from rods to cones, and the normal eye resolves the image and its color with utmost precision.

The investigations of Selig Hecht (5) defined the nature of the photoreceptor process whereby the rods and the cones of the retina respond to light of low and high intensities respectively and convert this light energy into nerve impulses which are carried to the brain via the optic nerve. It is not yet clear by what mechanism the brain again resolves these nerve impulses into the visual pattern which we actually "see" and which initiated the whole process.

Rhodopsin and Porphyropsin — The Visual Cycle

The biochemical basis of vision in dim light stems from the work of Franz Boll (6) who in 1876 discovered a red colored pigment in the rods of frog retina. This substance, first called visual red and later renamed visual purple or rhodopsin, was bleached in the light and resynthesized in the dark. Herein lies the center of activity of vision in dim light — this change initiated by the absorption of light by rhodopsin starts the chain of reactions responsible for the sensitivity of the rods to dim light.

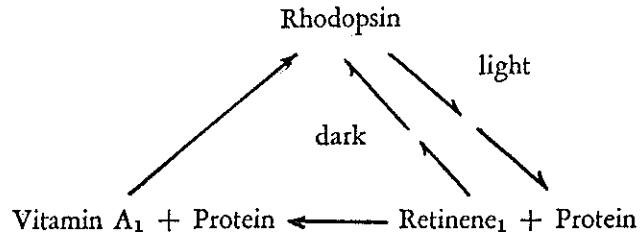
Although Boll discovered rhodopsin, Kuhne (7) must be credited for providing our basic knowledge of this substance. He visualized the retina and its pigment epithelium behaving not only like a photographic plate, but — "like an entire photographic workshop, in which the workmen continually renews the plate by laying on new light-sensitive material, while simultaneously erasing the old image."

Rhodopsin is found in the retinal rods of land and marine vertebrates. In the eyes of fresh-water vertebrates one finds the purple light sensitive pigment porphyropsin in place of rhodopsin. Both of these pigments are conjugated proteins, with each pigment containing a single type of the carotenoid prosthetic group. The protein moiety, called opsin, varies from animal to animal.

The processes which initiate vision must include: (a) the modification of the photosensitive pigment by light so as to cause excitation, (b) the removal of the excitation, and (c) the regeneration of the original pigment. In the light, rhodopsin (or porphyropsin) bleaches and the corresponding fall of the visual sensitivity to a constant, depressed level is "light adaptation." In the dark, the pigment is restored to its maximal concentration, and the accompanying rise of visual sensitivity to a maximum is known as "dark adaptation."

On exposure to light, the carotenoid portion of rhodopsin is first split from the protein, opsin, and is then degraded through orange intermediates to retinene, and then to colorless vitamin A. Similarly porphyropsin yields retinene$_2$ and vitamin A$_2$.

Although Wald (8) originally proposed the following cycle to explain the biochemistry of rod vision, he has since demonstrated that this scheme is an oversimplification of the actual process:



Rhodopsin may be synthesized either rapidly from retinene or much more slowly from vitamin A, with the result that one can observe correspondingly rapid and slow dark adaptation.

Since rhodopsin and porphyropsin have carotenoids as their prosthetic groups, one would expect them to have absorption spectra which are characteristic of these compounds. Indeed this physical property has been a most important factor in the elucidation of the mechanism of the rhodopsin cycle.

The absorption spectrum of rhodopsin has three bands (α, β and γ) only one of which is of primary concern here. This is the broad α-band with a maximum at a wave length of 500 mu and which is of chief concern for the spectral sensitivity of rod vision. Since vitamin A$_2$, the ultimate precursor of porphyropsin, has an absorption maximum (350 mu) somewhat higher than vitamin A (328 mu), one finds that the α-band of porphyropsin is about 522 mu or about 22 mu higher than rhodopsin.

On bleaching rhodopsin, the characteristic α-band at 500 mu is replaced by the spectrum of retinene$_1$ with its characteristic absorption maximum at 385 mu. (Retinene$_2$ may be similarly identified by its absorption maximum at 405 mu). Since the α-band of rhodopsin which is associated with the protein component of the molecule remains unchanged during the bleaching process, it is thought that light absorbed by the protein or γ-band is not available for bleaching.

Conversion of Rhodopsin to Retinene

It has been shown that there are two intermediate compounds involved in the conversion of rhodopsin to retinene$_1$: *lumi-rhodopsin* and *meta-rhodopsin.*

The reaction by which rhodopsin *is* converted to lumi-rhodopsin is a light reaction and is considered to be the only photochemical step in the rhodopsin cycle. This reaction was first elucidated by Broda and Goodeve (9) by irradiating rhodopsin at about — 78° C. Wald has shown that this step results primarily in a shift of the α-band of rhodopsin by about 5 mu toward shorter wave lengths (10).

Warming the solution of lumi-rhodopsin to about 20° C in the darkness causes a further change. The absorption band shifts 7–9 mu toward the blue. This product is called meta-rhodopsin. There is no formation of retinene$_1$ at this temperature, but if the solution of meta-rhodopsin is allowed to come to room temperature, a mixture of regenerated rhodopsin and retinene$_1$ plus protein in relatively equal amounts is obtained. If this solution is then exposed to light a second time at room temperature, all the regenerated rhodopsin will go over into retinene$_1$ plus protein.

It is interesting to note that dry rhodopsin on exposure to light is converted to lumi-rhodopsin which is then stable unless exposed to water which converts it to meta-rhodopsin.

Wald in a series of ingenious experiments has utilized this in making photographs with dry gelatin films of rhodopsin. This further exemplifies the photographic process by which the exposure of the film to light produces the *latent image* (the conversion of rhodopsin to lumi-rhodopsin) after which the film is *developed* simply by wetting (converts lumi-rhodopsin to meta-rhodopsin).

Inter-conversion of the Retinenes to the Vitamins A

Retinene$_1$ and retinene$_2$, derived from the prosthetic groups of rhodopsin and porphyropsin respectively, have been shown to be aldehydes: retinene$_1$ $C_{19}H_{27}CHO$ is the aldehyde of vitamin A$_1$ $C_{19}H_{27}OH$, while retinene$_2$ is the corresponding aldehyde of vitamin A$_2$ (11).

Now since an alcohol can be converted to an aldehyde by oxidation, it was soon shown that the transformation of vitamin A (an alcohol) to reti-

nene (the aldehyde) could be accomplished with ease, simply by passing a solution of vitamin A through a chromatographic column packed with an oxidizing agent (manganese dioxide) (12).

Although this fundamental act of oxidizing vitamin A to retinene (or reducing retinene to vitamin A) takes place in the eye it does this by the much more intricate mechanism of "biological oxidation and reduction."

Wald (13, 14) has shown that retinene$_1$ can be converted by a biological system to vitamin A_1 through the intervention of an enzyme called *retinene reductase* which reduces the aldehyde group of retinene$_1$ to the primary alcohol of vitamin A_1. To effect this conversion with retinal tissue, a water-soluble co-factor, present not only in retinal washings but also in boiled muscle juice, was needed.

In view of the fact that the nicotinamide containing co-factor, diphosphopyridine nucleotide (DPN) plays such a well known role in other biological oxidation — reduction reactions, it was an obvious choice to be tested in the system by which retinene is converted to vitamin A. Interestingly, when DPN itself was tested it had no effect on the conversion, but when the reduced form of the coenzyme was used (DPN–reduced) the reaction proceeded readily.

This system is pictured as follows:

$$C_{19}H_{27}CHO + \text{DPN-"reduced"} \xrightleftharpoons[\text{(retinene reductase)}]{} C_{19}H_{27}CH_2OH + \text{DPN}$$

retinene$_1$ Vitamin A_1

Two components of the system, DPN-"reduced" and the substrate, retinene$_1$ are well characterized, while the apoenzyme (retinene reductase) has not yet been isolated in pure form. It is extracted with dilute salt solutions from homogenized frog or cattle retinas, forming a clear, almost colorless solution. It can be precipitated from this by half-saturation with ammonium sulfate. Heating at 100° C destroys it within 30 seconds.

It is pertinent to note in passing that this system requires a second vitamin, niacin, in the form of DPN. Spaulding and Graham (15) have also reported that vitamin E protects the coenzyme in the above system. These are additional bits of evidence which point to the metabolic interrelationships by which the vitamins exert their biochemical function.

That retinene reductase acts in a rather non-specific way in the conversion of retinene$_1$, to vitamin A_1 is indicated by the fact that the apoenzyme from fish, like that of frogs or cattle, works equally well upon either of the

retinenes. It has further been shown (16) that crude alcohol dehydrogenase prepared from mammalian liver serves as well as retinene reductase. Yudkin (17) has shown too that a retinal enzyme system is capable of catalyzing the conversion of ethanol to acetaldehyde. It has been suggested (18) that retinene reductase may be identical with alcohol dehydrogenase.

The Oxidation of Vitamin A to Retinene

Although vitamin A_1 is in equilibrium with retinene$_1$, through the retinene reductase system, in which DPN-"reduced" functions as a coenzyme, the equilibrium lies far toward the side of the alcohol. To displace this reaction in the oxidative direction it is necessary to use an aldehyde trapping agent such as hydroxylamine, which condenses spontaneously with retinene$_1$ to form the oxime. In this process the *endergonic* oxidation of vitamin A_1 to retinene$_1$ is coupled with the *exergonic* trapping reaction. Such a trapping reaction plays a significant role, since to drive a reaction away from its equilibrium position work must be done; the trapping reaction provides this work.

The Synthesis of Rhodopsin from Vitamin A

Retinene condenses spontaneously with opsin to form rhodopsin *de novo* (19). It follows that this process can serve as a retinene-trapping reaction in the retina. Rhodopsin may therefore be synthesized by the retinene reductase system, coupled with the condensation of retinene with opsin to form rhodopsin. In this case, the oxidation of vitamin A_1 to retinene$_1$ would be the limiting reaction.

The initial energy requiring reaction in the visual process — the bleaching of rhodopsin to form lumi-rhodopsin — uses light as its source of energy. The reaction between opsin and synthetic retinene is a spontaneous, energy-yielding reaction. This regeneration of rhodopsin is maximum, however, only in the presence of additional added retinene. Wald (18) states "the main reason for this is that retinene$_1$, formed when rhodopsin bleaches, wanders away from its original sites of attachment to opsin, to couple with other groups on opsin and other molecules." The added retinene, in addition to "saturating" all such original sites of attachment, "so as to make adequate retinene$_1$ available at the sites concerned with rhodopsin synthesis," functions "simply to speed the synthesis of rhodopsin" since free opsin deteriorates rapidly in solution.

This synthesis of rhodopsin from retinene, coupled with the oxidation of vitamin A_1 to retinene$_1$ in the presence of an adequate trapping reagent provides a potential mechanism for the over-all synthesis of rhodopsin from vitamin A.

A series of experiments have been conducted by Wald and Hubbard (20, 21) in which they have systematically examined the effects on rhodopsin synthesis of factors which promote the oxidation of vitamin A.

In the initial experiments it was shown that isolated frog retinas and homogenates of retinal tissue form rhodopsin from vitamin A, albeit in small yields (i.e., about 10% as large as the *in vivo* dark reaction). As might be expected from what has been said, the addition of DPN to the retinal homogenate about doubled the yield of rhodopsin. It was also noted that the supplementation of the system with a homogenate of the pigment layers of the eye approximately doubled the yield of rhodopsin. It is evident that the pigment homogenate supplies something in addition to DPN since the yield of rhodopsin, when both are added to the system, is increased to about 40%. Additional work made it clear that the pigment homogenate alone was incapable of synthesizing rhodopsin when incubated with vitamin A_1 and DPN; further, no retinene reductase is contained in the pigment homogenate. It was demonstrated, however, that the pigment tissue supplies added vitamin A_1 to the system. Whether the pigment layer has functions in addition to supplying vitamin A must await further research.

To demonstrate further the importance of the retinene reductase — DPN system in the synthesis of rhodopsin, a system which provides a means of oxidizing DPN-"reduced" should make the synthesis function with optimum effectiveness. The enzyme chosen for this was the "succinoxidase" of heart muscle, which is a "particulate complex that includes all the components needed to transfer hydrogen from DPN to oxygen: cytochrome oxidase, the cytochromes, the riboflavin enzymes." It was found that the addition of "succinoxidase" to the retinal homogenate increased the yield of rhodopsin 35–50%.

Finally the validity of the proposed mechanism for the synthesis of rhodopsin was tested by the use of a model system which contained four known components: vitamin A_1, retinene reductase, DPN and the protein opsin. Because of its purity and known effectiveness, crystalline alcohol dehydrogenase was used in place of retinene reductase. The hypothesis that rhodopsin is synthesized by the oxidation of vitamin A_1 to retinene$_1$, coupled with the condensation of retine$_1$ with opsin, was confirmed by substantial rhodopsin synthesis in the above experiment.

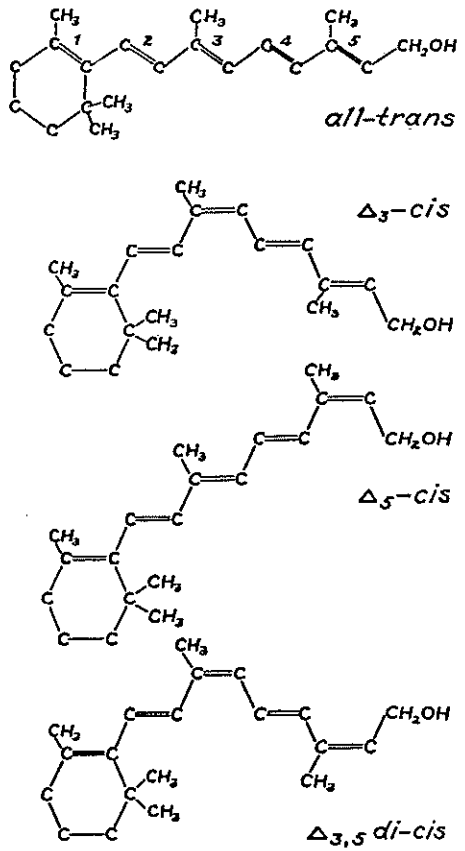
Cis-Trans Isomers of Vitamin A and Retinene and Vision

In the system just described, the investigators used a vitamin A concentrate derived from fish oils. An attempt to repeat this synthesis of rhodopsin, using crystalline synthetic vitamin A, resulted in failure (22). Since vitamin

A can exist in several stereoisomeric forms (see Figure 1), depending on the cis or trans arrangement about the double bonds, the question of which isomer is used for rhodopsin synthesis presented itself. The di-trans isomer is the most stable and is the form that prevails in the crystalline synthetic vitamin A. The △ 5-cis isomer has been prepared and has been named neovitamin A. The △ 3-cis isomer has also been prepared. The di-cis isomer has not, however, been absolutely identified. Since the active fish liver oils contain mixtures of di-trans and cis – isomers and the inactive synthetic vitamin A is the di-trans isomer, it follows that the cis-isomer is the one used in rhodopsin synthesis.

That there is easy inter-conversion between the isomers of vitamin A is indicated by the fact that crystalline vitamin A and neovitamin A, which are

FIGURE 1. STEREOISOMERS OF VITAMIN A



From Hubbard-Wald, *Science 115*, 60 (1952)

themselves ineffective as precursors of rhodopsin, are isomerized and become active when exposed to light in the presence of a trace of iodine.

It was soon shown that opsin was the "dominant isomer — specific component," since crystalline alcohol dehydrogenase acts with equal effectiveness on crystalline vitamin A and upon the natural mixture of the isomers. Retinenes prepared from di-trans and neovitamin A could not be converted to rhodopsin until they were isomerized by exposure to light.

Of even greater interest was the observation that retinene, prepared by the bleaching of rhodopsin in the dark with alcohol, was ineffective in the resynthesis of rhodopsin and did not become effective until isomerized by light.

Chase (23) and Chase and Smith (24) made the early observation that solutions of rhodopsin bleached with blue or violet light regenerated rhodopsin on subsequent incubation in the dark; whereas, rhodopsin exposed to yellow light failed to be regenerated. The reason for this is now evident: blue light isomerizes the inactive retinene produced by bleaching rhodopsin.

The absorption spectrum of retinene$_1$ lies at about 385 mu in aqueous solution, and extends into the violet and blue. Since retinene can only be isomerized by light that it can absorb, it follows that the blue light and not yellow light would isomerize it. Both blue and yellow light bleach rhodopsin to an inactive isomer of retinene, but only blue light isomerizes it. Thus, another step is added to the rhodopsin cycle.

It seems unlikely that this isomerization takes place to any large extent in the eye. Since vision continues very well in yellow, orange or red light, the active isomer must be provided in some other way. It has been proposed, therefore, that the inactive or trans isomer is continually removed into the circulation; while the active isomer is being supplied by the circulation from stores in the liver and from dietary sources. There is evidence to indicate that the body can isomerize vitamin A fed so that it is not necessary to have the active isomer provided in the diet.

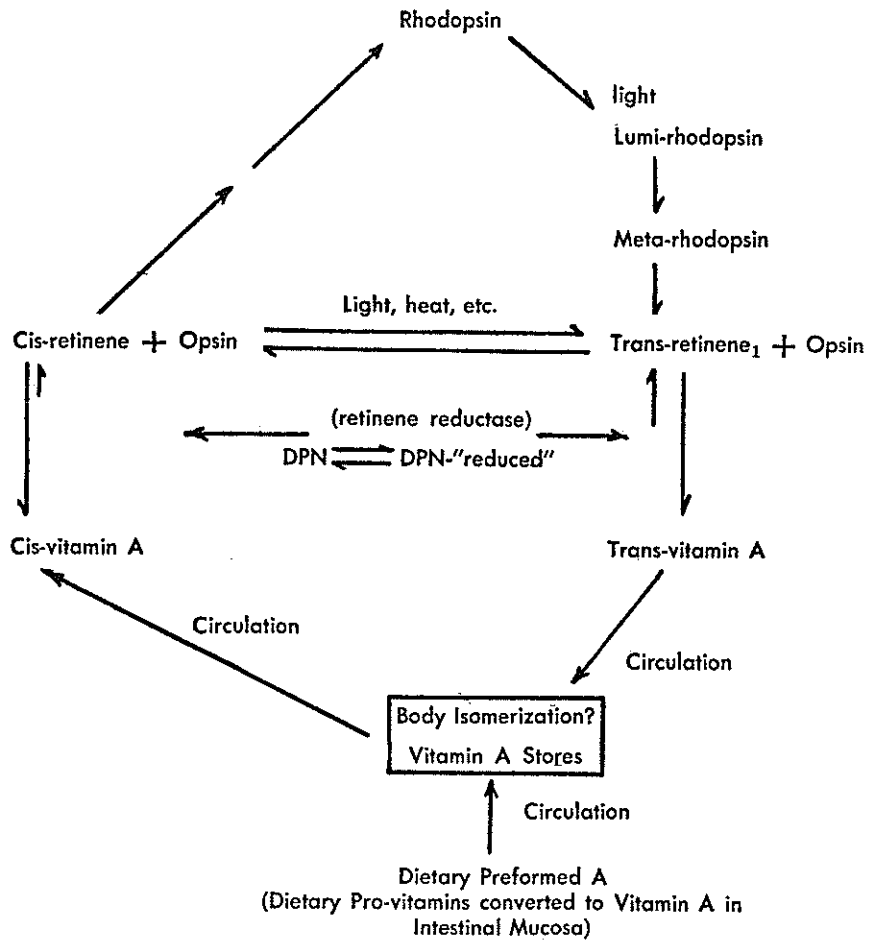
The over-all mechanism which summarizes the metabolic cycle involved in the synthesis of rhodopsin, the pigment responsible for vision in dim light, is shown in Figure 2.

Although there are yet gaps in our knowledge concerning the role of vitamin A in the process of vision, the mechanism proposed fits well with the experimental facts. Only additional research will reveal other mechanisms for rhodopsin synthesis, if such exist.

Summary

The best evidence available indicates that the following steps are involved in the so-called visual cycle: (a) In the presence of light, rhodopsin is bleached by a photochemical reaction to lumi-rhodopsin which is then convertible by a chemical reaction via meta-rhodopsin to trans-retinene$_1$ plus opsin; (b) trans-retinene$_1$ is removed as fast as it is formed by reduction to trans-vitamin A$_1$. Some trans-retinene$_1$ may be converted to cis-retinene$_1$ by blue light; (c) trans-vitamin A so formed goes to the circulation and in the body appears to be isomerized to the active cis-vitamin A; (d) the eye takes the active cis-vitamin A from the circulation which in turn derives it from the stores in the liver and from nutrition, and converts it to cis-retinene through the reaction of retinene reductase and DPN; (e) this reaction is

FIGURE 2. THE RHODOPSIN CYCLE



made to proceed in the oxidative direction because the condensation of retinene with opsin is exergonic, and so can serve as a retinene trapping reaction. Retinene is thus continually removed to form rhodopsin.

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